Peptidoglycan-associated lipoprotein (Pal) of Gram-negative bacteria: function, structure, role in pathogenesis and potential application in immunoprophylaxis

Renata Godlewska, Katarzyna Wiśniewska, Zbigniew Pietras & Elżbieta Katarzyna Jagusztyn-Krynicka

Department of Bacterial Genetics, Institute of Microbiology, Faculty of Biology, University of Warsaw, Warsaw, Poland

Correspondence: Elżbieta Katarzyna Jagusztyn-Krynicka, Department of Bacterial Genetics, Institute of Microbiology, Faculty of Biology, University of Warsaw, Miecznikowa 1, 02-096 Warsaw, Poland. Tel.: +48 22 5541216; fax: +48 22 5541402; e-mail: kjkryn@biol.uw.edu.pl

Abstract

The protein Pal (peptidoglycan-associated lipoprotein) is anchored in the outer membrane (OM) of Gram-negative bacteria and interacts with Tol proteins. Tol–Pal proteins form two complexes: the first is composed of three inner membrane Tol proteins (TolA, TolQ and TolR); the second consists of the TolB and Pal proteins linked to the cell's OM. These complexes interact with one another forming a multiprotein membrane-spanning system. It has recently been demonstrated that Pal is essential for bacterial survival and pathogenesis, although its role in virulence has not been clearly defined. This review summarizes the available data concerning the structure and function of Pal and its role in pathogenesis.

Introduction

Cell envelopes of Gram-negative bacteria consist of three layers: a lipid–protein inner (cytoplasmic) membrane (IM), an outer membrane (OM) composed mainly of lipopolysaccharides and proteins, and a thin rigid layer of peptidoglycan (polymeric chains of N-acetylmuramic acid and N-acetylglucosamine linked by short peptides) located in the periplasmic space. Peptidoglycan has the ability to interact with many proteins of the cell envelope, conditioning their stability. One of these is the peptidoglycan-associated lipoprotein (Pal), which constitutes part of the Tol–Pal protein system. In the majority of Gram-negative bacteria, the Tol–Pal complex is composed of five core proteins: TolQ, TolR, TolA, TolB and Pal. The Tol–Pal system forms a membrane-spanning multiprotein complex that exhibits structural and functional similarities to the MotA-B proteins (flagellar motor) and the TonB system (TonB, ExbB and ExbD) (Eick-Helmerich & Braun, 1989; Cascales et al., 2001). The TonB and Tol–Pal complexes participate in the penetration of phage particles into the cell (Riechmann & Holliger, 1997; Heilpern & Waldor, 2000) and in the translocation of colicins through the cell envelope (Webster, 1991; Bouveret et al., 1997, 1998).

The transcriptional organization of *Escherichia coli* genes encoding the Tol–Pal system has been characterized in detail. The genes comprise two adjacent operons that are positioned on the chromosome in the following order: (*ybgC–tolQ–tolR–tolA*)(*tolB–pal–ybgF*). Their transcription mainly occurs from two promoters located upstream of the 5′ ends of genes *ybgC* and *tolB*, although the presence of a long *ybgC–ybgF* transcript has been observed (Vianney et al., 1996; Muller & Webster, 1997). A similar organization is exhibited by the *tol–pal* genes in *Pseudomonas putida* (named *tol–oprL* in this microorganism) (Llamas et al., 2003a), *Actinobacillus actinomycetemcomitans* (now referred to as *Aggregatibacter actinomycetemcomitans*) (Paul-Satyaseela et al., 2006) and *Erwinia chrysanthemi* (Dubuisson et al., 2005). However, the *tol–pal* gene cluster is not universal. It has been identified in most sequenced genomes of Gram-negative bacteria except those of the spirochetes (*Treponema pallidum* and *Borellia burgdorferi*), *Rickettsia prowazeki* and *Neisseria meningitidis*. The gene cluster has not been found in archaea and in Gram-positive eubacteria

(Sturgis, 2001). The lowest level of sequence conservation relative to other cluster members is observed in the gene encoding TolA (Sturgis, 2001).

The Pal family of proteins is widespread in Gram-negative bacteria with sequence homologs identified in > 100 species (Parsons *et al.*, 2006).

Although ubiquitous in Gram-negative organisms, TolB and Pal are poorly conserved proteins; for example, among the sequences of 20 *Gammaproteobacteria* homologs, only *c.* 13% and *c.* 19% of TolB and Pal residues, respectively, are identical (Bonsor *et al.*, 2007).

Structure of Pal and the Tol–Pal complex

Pal of *E. coli* is synthesized as a precursor of 173 amino acids. This contains a signal sequence typical of lipoprotein precursors, including a characteristic LVAC motif cleaved by signal peptidase II during translocation through the cytoplasmic membrane. The first amino acid of the mature protein is a cysteine residue that is modified with lipids. The N-terminal end of the lipoprotein is hydrophobic and is responsible for anchoring the protein in the membrane. The second amino acid of the mature protein (+2), which determines the localization of Pal in the OM, is serine. Proper translocation of Pal through the cytoplasmic membrane and its correct localization are dependent on the Lol protein system (Liang *et al.*, 2005). The N-terminal region of Pal is likely to form a flexible tail, which binds the protein to the inner leaflet of the OM via lipid moieties linked to the cysteine at the +1 position (Clavel *et al.*, 1998; Cascales & Lloubes, 2004). The C-terminal region of Pal interacts with the cell wall peptidoglycan (Lazzaroni & Portalier, 1992). The structure of the complex between the periplasmic domain of Pal from *Haemophilus influenzae* (also known as P6) and the peptidoglycan precursor (PG-P) was solved by Parsons *et al.* (2006) using nuclear magnetic resonance (NMR) spectroscopy. They demonstrated that the majority of contacts are between conserved surface residues of Pal and the peptide region of PG-P. A specific region of Pal provides a pocket for the m-Dap residue of PG-P: a component of the cell wall present in the peptidoglycan layer of all Gram-negative bacteria. Thus, Pal forms a cross-bridge between the OM and peptidoglycan (Clavel *et al.*, 1998; Cascales & Lloubes, 2004; Parsons *et al.*, 2006).

TolB, which is located in the periplasm, contains an N-terminal region comprised of a five-stranded mixed β-sheet structure that sandwiches two major α-helices, and a C-terminal region with a six-bladed β-propeller domain. This domain competes with peptidoglycan to bind Pal (Abergel *et al.*, 1999; Carr *et al.*, 2000). TolB is considered to be an adapter protein that coordinates the association of OM/Pal with the peptidoglycan and the IM.

The other components of the Tol–Pal complex are three IM proteins TolQ, TolR and TolA, which associate via their transmembrane helices.

TolQ is a protein of 230 amino acids that contains three transmembrane domains (TolQI – amino acids 9–36, TolQII – 127–159, and TolQIII – 162–191) and two large cytoplasmic loops (Kampfenkel & Braun, 1993; Parsons *et al.*, 2008).

The 421-amino acid TolA protein is composed of three structural domains: the transmembrane TolAI domain (amino acids 1–47), TolAII (48–310) and TolAIII (311–421). TolAI, which anchors the protein in the cytoplasmic membrane, is separated from the globular periplasmic domain TolAIII by a periplasmic central domain (TolAII) with a high degree of α-helical structure (Derouiche *et al.*, 1999). The three domains are separated by segments rich in glycine residues, which provide a certain level of flexibility (Levengood *et al.*, 1991). The C-terminal domain of TolA is responsible for interaction with the N-terminal domain of colicin A (AT1) and phage g3p (Lubkowski *et al.*, 1999; Bouveret *et al.*, 2002; Bonsor *et al.*, 2007). The three-dimensional structure of free TolAIII was solved by Deprez *et al.* (2005) using NMR spectroscopy. They compared it with the available crystal structure of the g3pN1-TolAIII complex and found that the global fold of TolAIII is essentially the same in both the free form and the complex. Some changes in the secondary structure of TolAIII in the complex with g3pN1 were observed, especially in the α1 helix and β3 strand, which are involved in the interaction between the proteins (Deprez *et al.*, 2005).

It has been postulated that TolR (142-amino acid protein), besides the membrane-anchoring segment (TolRI), possesses a short central domain (TolRII) and a periplasmic C-terminal domain (TolRIII) that interacts with the cytoplasmic membrane (Lazzaroni *et al.*, 1995; Journet *et al.*, 1999). However, Parsons *et al.* (2008) solved the NMR structure of the homodimeric periplasmic domain of TolR from *H. influenzae* and proposed a new structural model. According to this model, TolR has only two domains (transmembrane and periplasmic) and a C-terminal tail. In contrast to its homolog ExbD, the TolR periplasmic domain forms a dimer and its C-terminal tail is oriented toward the IM and probably interacts with TolQ (Parsons *et al.*, 2008).

TolQ and TolR are homologous to ExbB and ExbD of the TonB system, respectively, while TolA is a homolog of TonB (Cascales *et al.*, 2001; Zhai *et al.*, 2003). Amino acid sequence analyses of the transmembrane domains of the TolQ and TolR proteins revealed their structural and functional similarity not only to the ExbB and ExbD proteins but also to MotA and MotB of the flagellar apparatus. Similarities between the C-terminal 'hairpin' structures of TolQ, ExbB and MotA as well as between the N-terminal domains of the TolR, ExbD and MotB transmembrane proteins were identified. A common feature of these three protein systems

is their ability to exploit the transmembrane electrochemical gradient as a source of energy (Cascales *et al.*, 2001).

The Tol–Pal proteins form two complexes (Fig. 1). The first is a complex of TolQ, TolR and TolA with a 4–6 : 2 : 1 stoichiometry, anchored in the cytoplasmic membrane (Cascales *et al.*, 2001; Zhang *et al.*, 2008). TolAI interacts with the first TolQ transmembrane domain and with the TolRI transmembrane domain (Derouiche *et al.*, 1995; Germon *et al.*, 1998). The first and third transmembrane domains of TolQ probably also interact with each other (Lazzaroni *et al.*, 1995). The TolQIII domain interacts with the TolR (TolRI) transmembrane region and with the TolR dimer (Walburger *et al.*, 2002).

The second complex is composed of TolB and Pal linked to the OM. Pal is anchored in the OM and interacts with the C-terminal domain of the periplasmic TolB, which possesses a β-propeller structure (Bouveret *et al.*, 1995; Ray *et al.*, 2000). In addition, Pal interacts with TolA and other cell envelope proteins such as OmpA and Lpp. All of these interactions are independent of each other (Cascales & Lloubes, 2004).

The two Tol–Pal complexes interact with each other via the C-terminal domain of TolA, which is able to bind Pal as well as TolB (Carr *et al.*, 2000; Cascales *et al.*, 2000, 2002; Lloubes *et al.*, 2001). Dubuisson *et al.* (2002) obtained

suppressors of *tolA* mutations in the *tolB* gene and confirmed the interaction between TolAIII and the N-terminal domain of TolB. Experiments using the yeast two-hybrid system and cross-linking assays with purified proteins allowed Walburger *et al.* (2002) to identify the domains of TolA responsible for the interactions with TolB and YbgF.

In vitro analysis of the interactions between the components of these complexes as well as the resolution of the three-dimensional structure of TolB and of the complex formed by the Pal periplasmic domain and the PG-P permitted the precise localization of regions of Pal that are responsible for forming protein complexes (Abergel *et al.*, 1999; Cascales & Lloubes, 2004; Parsons *et al.*, 2006). By identifying suppressor mutants, Ray *et al.* (2000) characterized the regions responsible for the interaction between TolB and Pal. The region between amino acid residues 94–121 of Pal is responsible for its interactions with peptidoglycan and TolB, but Pal cannot bind both molecules at the same time (Lazzaroni & Portalier, 1992; Clavel *et al.*, 1998; Bouveret *et al.*, 1999).

An elegant study by Bonsor *et al.* (2007) analyzing the mechanism by which nuclease colicins destabilize the Tol–Pal complex to facilitate their entry into the cell revealed many details of Pal–TolB interaction. They found that there is a substantial overlap between the colicin E9 and Pal
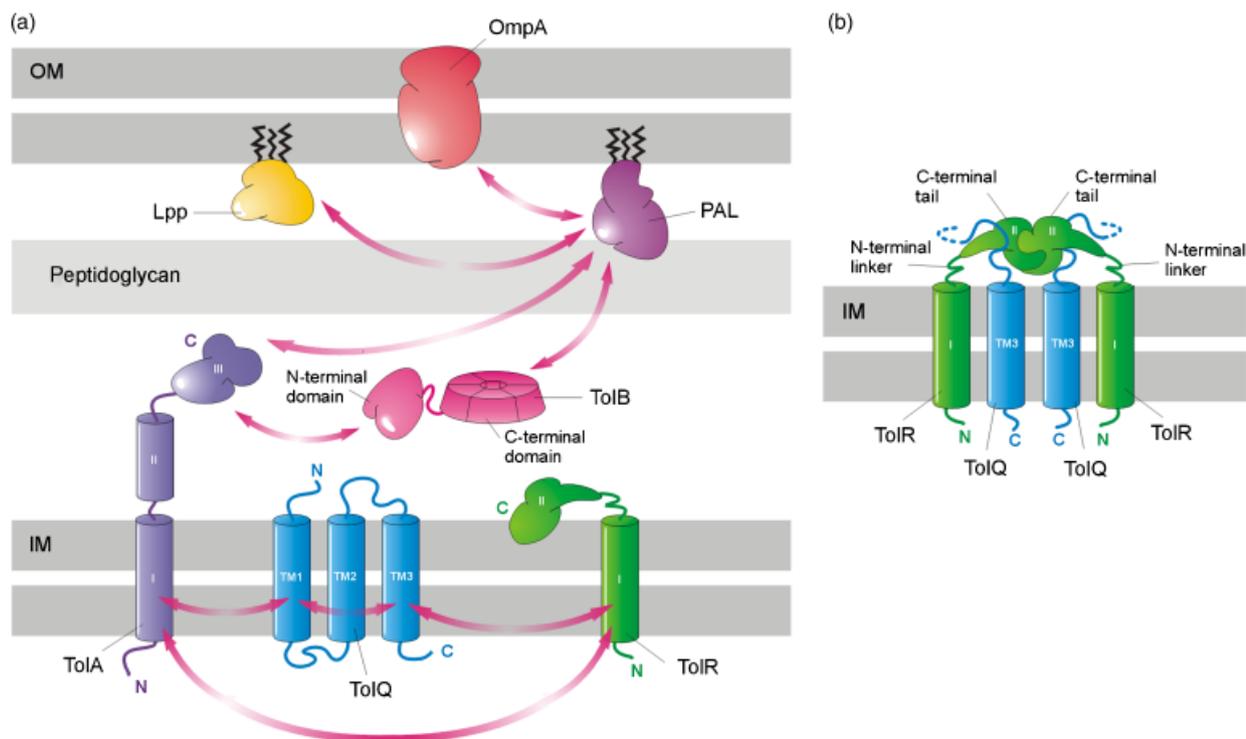

Fig. 1. (a) Location and interactions of Tol–Pal proteins in the cell envelope of Gram-negative bacteria. The various interactions are indicated by arrows (see details in the text). (b) Hypothetical model of the TolR interaction with the membrane and TolQ. Only one TolQ transmembrane domain (TM3) was shown to clarify the scheme.

binding sites on TolB. The natively disordered region of colicin E9, which is part of its translocation domain, interacts strongly with TolB, resulting in destabilization of the Tol–Pal complex. TolB binding with Pal occurs through an induced-fit mechanism in which both proteins undergo conformational changes (Bonsor *et al.*, 2007). In addition, the interactions of TolB with other proteins (OmpA and Lpp) are dependent on its interaction with Pal (Walburger *et al.*, 2002). The interaction of Pal with the OM protein OmpA occurs via a region between amino acid residues 44 and 61, containing a conserved VYF motif. The region of Pal that is responsible for binding to the TolA C-terminal domain (TolB interacts with the same TolA domain) was also identified. It is constituted by 30 C-terminal amino acid residues of Pal, which contain a conserved SYG(K/E) motif termed the TolA box, plus the KNRR motif. Deletion of the SYG(K/E) motif results in loss of the interaction between Pal and TolA, while the KNRR motif has no influence on this process (Cascales & Lloubes, 2004). Cascales *et al.* (2000) demonstrated that the TolA–Pal interaction is dependent on the proton motive force (PMF). Interactions between proteins of the Tol–Pal system as well as those with other proteins (OmpA and Lpp), especially TolA–Pal and TolQ–TolR, result in the formation of transenvelope bridges linking the IMs and OMs, and the peptidoglycan layer (Cascales *et al.*, 2000; Walburger *et al.*, 2002).

Physiological function of the Tol–Pal system in the cells of Gram-negative bacteria

Inactivation of *ybgC* and *ybgF* genes within the *tol–pal* gene cluster of *E. coli* does not cause noticeable phenotypic changes in the cell. YbgC is a cytoplasmic protein with thioesterase activity (Zhuang *et al.*, 2002; Gully & Bouveret, 2006), while YbgF is a protein of unknown function located in the periplasm. Mutations in the remaining *tol–pal* genes evoke various physiological changes such as hypersensitivity to detergents, bile salts and several antibiotics. Other effects include the release of periplasmic proteins from the cell, impaired motility, aberrant cell division and the induction of colanic acid production (Webster, 1991; Clavel *et al.*, 1996; Lazzaroni *et al.*, 1999; Llamas *et al.*, 2000). Moreover, *tolA*, *tolQ*, *tolR* and *pal* mutants of *E. coli* are characterized by their ability to produce large amounts of outer membrane vesicles (OMVs) (Bernadac *et al.*, 1998; Balsalobre *et al.*, 2006). The aforementioned phenotypic changes produced by mutations in the *tol–pal* genes suggest that the encoded proteins are involved in maintaining the proper structure and function of the cell envelope.

The findings of other studies also suggest the involvement of the Tol–Pal system in the biogenesis and/or transport of lipopolysaccharide components (Gaspar *et al.*, 2000; Vines

et al., 2005). The TolA and Pal proteins modulate the structure of the surface O-antigen by playing a role in the translocation of its subunits through the OM. Damage to bacterial cell envelopes caused by an abnormal O-antigen structure results in the activation of regulatory cascades (extracytoplasmic stress response pathways). Increased expression of the *degP* gene, encoding a chaperone protein (protease) has been observed in response to the extracytoplasmic stress caused by inactivation of the *tolA* and *pal* genes (Gaspar *et al.*, 2000; Vines *et al.*, 2005).

Gerding *et al.* (2007) described the involvement of proteins from the Tol–Pal complex in the formation of cell envelopes in daughter cells. They found that Tol–Pal proteins accumulate at the site of cell constriction, where the septum separating the daughter cells is formed. Cells of *tolA$^-$*, *pal$^-$* and *tolQ$^-$pal$^-$* mutants exhibit an increased release of vesicles from the OM. Interestingly, 90% of these vesicles also localize at the site of cell division and at the poles of the dividing cell. These authors proposed a hypothetical model for the role of the Tol–Pal complex in the invagination of the OM and in connecting the OM with the IM and peptidoglycan layer during septum formation (Gerding *et al.*, 2007). As mutations in the Tol–Pal proteins are not always lethal, it is likely that their role may be partially substituted by other OM peptidoglycan-associated proteins, for example Lpp or OmpA.

Tol–Pal proteins have also been shown to play a role in the uptake/transport of compounds through the cytoplasmic membrane. *Pseudomonas putida tol-oprL* gene mutants do not grow or grow very weakly on media containing certain compounds as the sole carbon source (e.g. arginine, fructose, glycerol and saccharose). This is due to perturbations in compound transport and not the abnormal structure/activity of the OM porins. Similar results were obtained for *E. coli* and *Pseudomonas aeruginosa tol–pal* gene mutants (Llamas *et al.*, 2003b). Llamas *et al.* (2003b) also reported that the PMF in *P. putida tolA, tolB* and *oprL* mutants is similar to that produced by the wild-type strain, but is lower in *tolQ* and *tolR* mutants.

Role of Pal in the process of pathogenesis

Role of lipoproteins in pathogenesis

The results of recent studies indicate a significant role for lipoproteins in the pathogenesis of bacterial infections caused by species such as *Yersinia pestis* (Sha *et al.*, 2008), *P. aeruginosa* (Rau *et al.*, 2006), *Salmonella enterica* sv. Typhimurium (Sha *et al.*, 2004), *N. meningitidis* (Delgado *et al.*, 2007), *Borrelia burgdorferi* (Porcella & Schwan, 2001) and *Mycobacterium tuberculosis* (Neufert *et al.*, 2001). Bacterial lipoproteins belong to the so-called pathogen-associated molecular patterns modulating the host immune response.
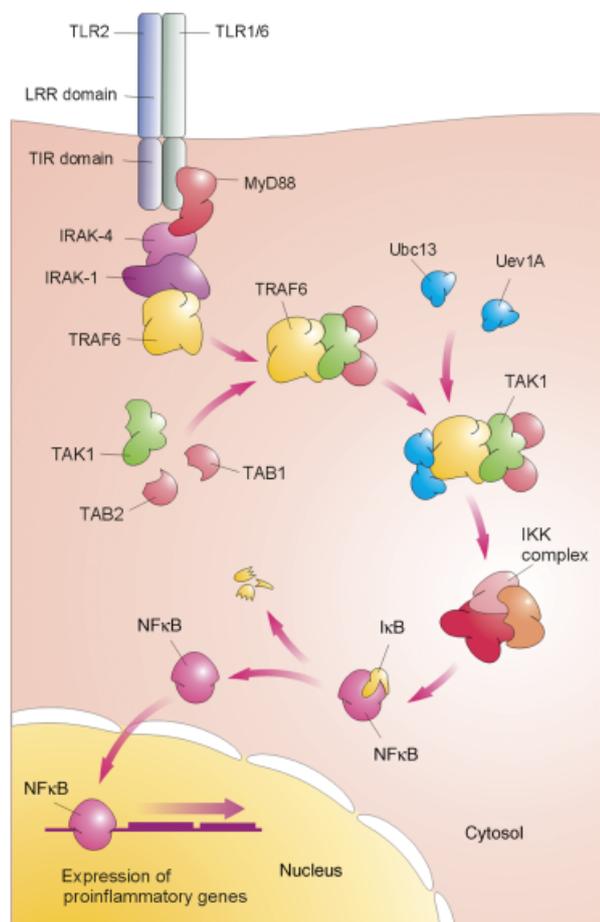
Fig. 2. Signaling cascade in antigen-presenting cells induced by stimulation of TLR2 receptors. Activated TLR2 associates with myeloid differentiation primary-response protein 88 (MyD88) and then recruits of interleukin-1 receptor (IL-1R)-associated kinase 4 (IRAK4) and IRAK1. TNF-receptor-associated factor 6 (TRAF6) binds IRAK1 and then dissociates to form a complex with transforming growth factor-β-activated kinase 1 (TAK1), TAK1-binding protein 1 (TAB1) and TAB2. This complex is required for the ubiquitylation of TRAF6 and for the activation of the kinase activity of TAK1, which then leads to the phosphorylation of components of the IKK complex [the inhibitor of NF-κB (IκB)-kinase complex] and gives rise to downstream activation of NF-κB. Ubc13, ubiquitin-conjugating enzyme 13; Uev1A, ubiquitin-conjugating enzyme E2 variant 1; NF-κB, nuclear factor-κB.

They are recognized by Toll-like receptors (TLRs) located mainly on the surface of antigen-presenting cells, for example macrophages and dendritic cells. This leads to the activation of a signal transduction cascade (Fig. 2), which results in proinflammatory cytokine gene transcription. Lipoproteins are recognized by class TLR2 receptors (Aliprantis et al., 1999) in a manner dependent on TLR6 or TLR1, which participate in the induction of immune responses to diacylated (Takeuchi et al., 2001) or triacylated molecules (Takeuchi et al., 2002), respectively. For certain

lipoproteins, the signaling cascades are stimulated independently of TLR1 and TLR6 (Buwitt-Beckmann et al., 2006).

Some Gram-negative microorganisms have the ability to secrete lipoproteins to the extracellular environment. Among these is Pal, which is released into the bloodstream during infection, and this process contributes to the development of septic shock (Hellman et al., 2002; Liang et al., 2005).

Role of Pal in pathogenesis

Escherichia coli

During experimentally induced sepsis in mice caused by *E. coli* infection, significant amounts of Pal are released into the bloodstream. The addition of purified Pal (in amounts of 10 μg) to the C3H/HeJ mouse cell line resulted in the induction of immune responses in macrophages and spleen lymphocytes similar to those observed following lipopolysaccharide application (increased production of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and nitric oxide), and induced early sepsis symptoms. The administration of Pal to D-galactosamine-sensitized mice at doses of 1, 10 and 100 μg was found to cause death with a lethal dose 50% of 3 μg per mouse (Hellman et al., 2002; Liang et al., 2005).

The infection of C3H/HeN mice with *E. coli* strains exhibiting decreased expression of the *pal* gene or producing a truncated form of the protein led to the development of a mild form of sepsis characterized by increased survival of infected animals and lower levels of IL-6 in the blood (Hellman et al., 2002). Bacteria with a mutated *pal* gene might be less efficient in inducing sepsis for several reasons. Possibly, disorders in the functioning of the bacterial cell envelope caused by structural alteration or deficiency of Pal aid the host immune system to overcome the infection. The effect of passive immunization with mono- and polyclonal anti-Pal antibodies has been tested in three different mouse models of sepsis (cecal ligation and puncture, an infected burn model, and an infected fibrin clot model mimicking peritonitis). However, these trials indicated that this type of strategy does not have any protective/therapeutic effect (Valentine et al., 2006).

Aggregatibacter actinomycetemcomitans

Antibodies interacting strongly with Pal are present in the serum of patients infected with *A. actinomycetemcomitans* (Norskov-Lauritsen & Kilian, 2006) and suffering from paradontosis. These antibodies cross-react with putative Pal proteins from other microorganisms residing in the oropharyngea (*Aggregatibacter aphrophilus*, *Aggregatibacter paraphrophilus*, *H. influenzae*). A humoral anti-Pal immune

response in patients with paradontosis may be one of the factors that induces the illness symptoms (Ihalin *et al.*, 2006; Paul-Satyaseela *et al.*, 2006). *Aggregatibacter actinomycetemcomitans* biofilms and planktonic cells release free soluble material independently of the cell vesicles (Oscarsson *et al.*, 2008). Along with a GroEL-like protein, Pal was found to be one of the major protein antigens released in this way. This process is likely to have biological significance because free proteins can readily cross biological barriers that are otherwise impermeable to whole bacterial cells or even membrane vesicles. Further studies are required to elucidate the mechanism of the release of Pal from bacterial cell envelopes (Karched *et al.*, 2008). Oscarsson *et al.* (2008) reported that free-soluble material released by wild-type and *pal*-mutant planktonic *A. actinomycetemcomitans* cells induce similar cytokine responses in whole blood. However, in a previous study, these authors found that purified Pal stimulates proinflammatory cytokines in whole blood (Karched *et al.*, 2008). This discrepancy was attributed to the enhanced release of lipopolysaccharides by the *pal*-deficient mutant strain (Oscarsson *et al.*, 2008).

Haemophilus ducreyi

Haemophilus ducreyi is the etiological agent of chancroid, an infectious sexually transmitted genital ulcer disease (Bauer & Spinola, 2000; Bong *et al.*, 2002). Studies carried out on healthy individuals showed that in comparison with the wild-type strain, an *H. ducreyi pal* mutant caused less profound skin changes that rarely transformed into ulcerating forms and fewer bacteria were isolated from biopsy samples taken from the forming wound. This finding could be due to the decreased survival of *pal* mutants or a reduced ability to induce an immune response and the lower numbers of neutrophils, lymphocytes, macrophages or dendritic cells recruited to the site of infection (Fortney *et al.*, 2000).

Erwinia chrysanthemi

It has been demonstrated that the Tol–Pal complex participates in the virulence of *E. chrysanthemi*: a pathogen causing soft rot in many plant species including vegetables and ornamentals (Dubuisson *et al.*, 2005). Bacteria of this species colonize the parenchymatous tissues and break down the plant cell wall material using pectinolytic enzymes, while oligosaccharides, the products of pectin degradation, are utilized as a carbon source. Dubuisson *et al.* (2005) obtained *E. chrysanthemi ybgC, tolQ, tolA, tolB, pal* and *ybgF* gene mutants, characterized by increased sensitivity to bile salts. Bacteria with mutated *tolQ, tolA, tolB* and *pal* genes survived only in an environment with increased levels of sugars and osmoprotectants. Changes in cell morphology and abnor-

mal cell division were observed, with the most severe defects seen in *pal* and *tolB* mutant cells. The virulence of the mutant strains was examined using potato tuber and chicory leaf models. *In vivo* all of the obtained mutants damaged plant tissues to a lower extent than the wild-type strain. They also exhibited reduced motility, most probably due to abnormalities in flagellum synthesis. Moreover, apart from the *ybgF* mutant (which showed increased pectinolytic enzyme activity), the investigated mutants showed decreased pectin-degrading activity: the main virulence factor of *E. chrysanthemi* (Dubuisson *et al.*, 2005).

Vibrio cholerae

The mechanism by which Tol–Pal proteins participate in the process of *V. cholerae* infection (pathogen colonizing the small intestine) has been characterized recently. Cholera exotoxin is the main *V. cholerae* virulence factor that stimulates the secretory response in epithelial cells leading to diarrhea. The toxin is encoded by the *ctxAB* operon found in the genome of phage CTXΦ. The acquisition of phage genes in the course of evolution was an essential step in the development of *V. cholerae* pathogenicity. Mutants in the *tolQRA* genes created by Heilpern & Waldor (2000) were defective in the transport of phage CTXΦ DNA through the periplasm. During phage injection into the bacterial cell, the periplasmic domain of the TolA protein binds the minor coat protein pIII and serves as a coreceptor for phage entry (Riechmann & Holliger, 1997). Mutation of any of the *V. cholerae tolQRA* genes was found to decrease the infection ability of the CTXΦ phage, but did not confer full resistance (Heilpern & Waldor, 2000). Mutation of the *tolB* gene had no influence on phage injection into the *V. cholerae* cells.

Application of Pal in immunoprophylaxis

All Omp18 (Pal) proteins studied so far are highly immunogenic, which makes them attractive antigens for the production of subunit vaccines.

Haemophilus influenzae

Atypical *H. influenzae* [nontypeable *H. influenzae* (NTHi)] strains have been investigated as candidates for the development of subunit vaccines based on Pal. NTHis are commonly occurring pathogens of the respiratory tract in both children and adults. Otitis media, one of the most serious childhood illnesses caused by NTHi is currently treated with antibiotic therapy (Murphy *et al.*, 2006). Considerable research effort has been put into developing a vaccine against NTHi. So far, the most intensively studied vaccine candidate is lipoprotein P6 (16 kDa) from the Pal family. P6 is highly conserved among all strains of this species. Murphy *et al.* (2006) showed that a P6 gene mutant exhibits

abnormal cell morphology and decreased survival. The P6 protein was found to activate the transcription factor nuclear factor-κB in eukaryotic HM3 cells by stimulating TLR2 receptors, leading (among other responses) to increased *muc5ac* gene transcription, followed by increased mucin production. The *muc5ac* gene plays a significant role in infectious diseases of the respiratory tract (Chen *et al.*, 2004). Stimulation of TLR receptors of human macrophages by P6 increases the production of proinflammatory cytokines, especially IL-8, TNF-α and IL-10 (Berenson *et al.*, 2005). In addition, treatment with protein P6 activates T CD4$^+$ lymphocyte proliferation and the expression of genes encoding certain cytokines (Kodama *et al.*, 2006).

Intravenous and mucosal (intranasal) immunization of experimental animals (mouse, rat and chinchilla) with the P6 protein induces a strong specific immune response (e.g. production of specific mucosal type sIgA antibodies) and protects against infection (Badr *et al.*, 1999; Kodama *et al.*, 2006). The immunogenicity of Pal depends on a 15-amino acid-long peptide (residues 41–55 of the P6 protein sequence). The immunization of mice with this synthetic peptide produced an immune response of the same magnitude as administration of the complete protein and hastened the amelioration of the infection. Modifications of the amino acid sequence showed that effective protection of the immunized animal was afforded only when the peptide induced both lymphocyte B and T responses. This confirmed the importance of both humoral and cellular immune responses in the defensive reaction of the organism against *H. influenzae* (McMahon *et al.*, 2005).

In light of the high number of otitis media cases among children below the age of 2, the possibility of transmitting maternal anti-Pal antibodies to offspring was examined in experimental animals. Yamauchi *et al.* (2006) showed that female mice transmit specific IgG antibodies against the P6 protein to their offspring. These antibodies were detected in the serum of young directly after birth and in the mother's milk, and persisted for longer in offspring that were breast-fed by immunized mothers (Yamauchi *et al.*, 2006).

Campylobacter jejuni

The *cjaD* gene of *C. jejuni* cj0113 (*C. jejuni* NCTC 1168) was identified by immunological screening of a *C. jejuni* genomic DNA library using anti-*Campylobacter* rabbit serum. This gene encodes an 18-kDa protein conserved among many *Campylobacter* serotypes, which potentially belongs to the Pal family (*in silico* analysis). This protein contains a signal sequence characteristic of lipoprotein precursors reacting not only with anti-*Campylobacter* rabbit serum but also with the serum of patients infected with different *C. jejuni* serotypes (Burnens *et al.*, 1995; Konkel *et al.*, 1996; Pawelec *et al.*, 2000; Wyszynska *et al.*, 2002). The analysis of

proteins from different cellular fractions using rabbit serum raised against rCjaD (fusion protein CjaDx6His expressed in *E. coli*) demonstrated that CjaD is located in the OM. Proteomic experiments have also shown that *Campylobacter* Pal (Omp18) is a cell membrane-associated protein and is immunogenic during human infection (Cordwell *et al.*, 2008). The *cjaD* gene was cloned in an avirulent *S. enterica* sv. Typhimurium strain using plasmids from the Asd series that are used to express foreign antigens for vaccine studies. Oral immunization of chickens with the avirulent *cjaD*-carrying *Salmonella* strain induced specific class IgG and IgA immune responses in the blood serum and mucus, respectively (E.K. Jagusztyn-Krynicka *et al.*, unpublished data). Until now, attempts to obtain a *C. jejuni cjaD* mutant have been unsuccessful, which indicates that Pal is essential for cell survival. This feature makes it an attractive potential candidate for vaccine construction.

Legionella pneumophila

Legionella pneumophila is the causative agent of serious respiratory tract diseases, often having the character of local epidemics. This infection is especially dangerous in immunocompromised individuals, for example the elderly or cancer therapy patients. The application of lipoprotein Pal in both the diagnosis and the immunoprophylaxis of legionellosis has been studied. Kim *et al.* (2003) showed that anti-Pal antibodies effectively detect the presence of *Legionella* spp. in urine samples of guinea pigs, giving almost no false positive results. *Legionella pneumophila* is an intracellular pathogen able to multiply in macrophages. As a result, the humoral response developed by patients vaccinated against *Legionella* sp. was found to be inefficient in combating this pathogen. Yoon *et al.* (2002) developed a DNA vaccine based on the *pal* gene and compared its efficiency with immunization with purified recombinant Pal. They observed that intravenously administered Pal is a strong inducer of the humoral response due to the activation of Th2 lymphocytes, but it does not evoke an efficient cellular response. In contrast, intramuscular administration of a plasmid expressing the *pal* gene from a eukaryotic promoter was found to induce a mainly Th1-type response as well as a strong cytotoxic response, indicating the potential application of Pal-encoding DNA as a vaccine against legionellosis (Yoon *et al.*, 2002).

Application of OMVs in medicine

Many types of Gram-negative bacteria constitutively secrete OMVs into the extracellular milieu (Kadurugamuwa & Beveridge, 1995, 1996). Compared with wild-type cells, *tol–pal* gene mutants generally produce increased amounts of OMVs (Bernadac *et al.*, 1998; Kuehn & Kesty, 2005; Lee *et al.*, 2007). Although the role of the Tol–Pal complex in

OMV formation remains to be elucidated, the creation of cells that overproduce OMVs owing to a defect in the Tol–Pal system may be of great value in combating infectious diseases. Such systems may be used as vectors delivering antibiotics to eukaryotic cells or for vaccine development (Henry *et al.*, 2004; Ferrari *et al.*, 2006).

Neisseria meningitidis, a Gram-negative bacterium categorized into five serogroups, is a major cause of meningitis and sepsis. Effective conjugated capsular polysaccharide-based vaccines have been developed to protect against infection by four serogroups: A, C, W-135 and Y. However, there is no effective vaccine against B strains (MenB), which are very common in some parts of the world. Great effort has recently been put into the development of an anti-MenB vaccine based on OMVs containing OM proteins. Detergent-extracted OMVs (DOMVs) have been tested in phase III clinical trials with promising results (O'Hallahan *et al.*, 2004). Two important prerequisites for the success of this strategy are a means to control the composition of OMVs and a reproducible method for their production (Vipond *et al.*, 2006). Ferrari *et al.* (2006) performed a proteomic comparison of detergent-derived MenB OMVs (DOMVs) with m-OMVs spontaneously released by a MenB strain carrying a deletion of lytic transglycosylase (Δ*gna33*), an enzyme homologous to *E. coli* MltA. Their analysis showed that m-OMVs contain mainly OM proteins whereas about 70% of DOMV proteins are cytoplasmic or from the IM (Ferrari *et al.*, 2006).

Conclusions

Studies on Pal and the Tol–Pal system of *E. coli* have been conducted for over 30 years. They have resulted in a significant understanding of the structure and interactions of these proteins and their involvement in maintaining the integrity of the cell envelope. Besides its structural functions, Pal also appears to play an important role in the pathogenesis of certain Gram-negative bacteria. Despite numerous studies on the potential use of Pal and its synthetic derivatives as vaccines against various pathogens, none of these vaccine prototypes have so far entered the phase of clinical trials. Although Pal is found in many Gram-negative bacteria, immunization against diverse pathogens is likely to require the application of different forms of administration (DNA vaccines vs. subunit protein vaccines) and the use of different adjuvants or carrier strains. Depending on the immunization strategy, the same protein may induce an immune response dependent on Th1 and/or Th2 lymphocytes. Studies aimed at using OMVs as carriers of prophylactic agents have also provided interesting data. Developments in these fields of research are likely to be dependent on further studies on the functional mechanisms of Pal–Tol complexes. Detailed knowledge about the struc-

ture of Pal–Tol complexes might also facilitate the design of new antibacterial drugs.

Acknowledgements

References

Abergel C, Bouveret E, Claverie JM, Brown K, Rigal A, Lazdunski C & Benedetti H (1999) Structure of the *Escherichia coli* TolB protein determined by MAD methods at 1.95 Å resolution. *Structure* **7**: 1291–1300.

Aliprantis AO, Yang RB, Mark MR *et al.* (1999) Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. *Science* **285**: 736–739.

Badr WH, Loghmanee D, Karalus RJ, Murphy TF & Thanavala Y (1999) Immunization of mice with P6 of nontypeable *Haemophilus influenzae*: kinetics of the antibody response and IgG subclasses. *Vaccine* **18**: 29–37.

Balsalobre C, Silvan JM, Berglund S, Mizunoe Y, Uhlin BE & Wai SN (2006) Release of the type I secreted alpha-haemolysin via outer membrane vesicles from *Escherichia coli*. *Mol Microbiol* **59**: 99–112.

Bauer ME & Spinola SM (2000) Localization of *Haemophilus ducreyi* at the pustular stage of disease in the human model of infection. *Infect Immun* **68**: 2309–2314.

Berenson CS, Murphy TF, Wrona CT & Sethi S (2005) Outer membrane protein P6 of nontypeable *Haemophilus influenzae* is a potent and selective inducer of human macrophage proinflammatory cytokines. *Infect Immun* **73**: 2728–2735.

Bernadac A, Gavioli M, Lazzaroni JC, Raina S & Lloubes R (1998) *Escherichia coli* tol–pal mutants form outer membrane vesicles. *J Bacteriol* **180**: 4872–4878.

Bong CT, Bauer ME & Spinola SM (2002) *Haemophilus ducreyi*: clinical features, epidemiology, and prospects for disease control. *Microbes Infect* **4**: 1141–1148.

Bonsor DA, Grishkovskaya I, Dodson EJ & Kleanthous C (2007) Molecular mimicry enables competitive recruitment by a natively disordered protein. *J Am Chem Soc* **129**: 4800–4807.

Bouveret E, Derouiche R, Rigal A, Lloubes R, Lazdunski C & Benedetti H (1995) Peptidoglycan-associated lipoprotein-TolB interaction. A possible key to explaining the formation of contact sites between the inner and outer membranes of *Escherichia coli*. *J Biol Chem* **270**: 11071–11077.

Bouveret E, Rigal A, Lazdunski C & Benedetti H (1997) The N-terminal domain of colicin E3 interacts with TolB which is involved in the colicin translocation step. *Mol Microbiol* **23**: 909–920.

Bouveret E, Rigal A, Lazdunski C & Benedetti H (1998) Distinct regions of the colicin A translocation domain are involved in

the interaction with TolA and TolB proteins upon import into *Escherichia coli*. *Mol Microbiol* **27**: 143–157.

Bouveret E, Benedetti H, Rigal A, Loret E & Lazdunski C (1999) *In vitro* characterization of peptidoglycan-associated lipoprotein (PAL)-peptidoglycan and PAL–TolB interactions. *J Bacteriol* **181**: 6306–6311.

Bouveret E, Journet L, Walburger A, Cascales E, Benedetti H & Lloubes R (2002) Analysis of the *Escherichia coli* Tol–Pal and TonB systems by periplasmic production of Tol, TonB, colicin, or phage capsid soluble domains. *Biochimie* **84**: 413–421.

Burnens A, Stucki U, Nicolet J & Frey J (1995) Identification and characterization of an immunogenic outer membrane protein of *Campylobacter jejuni*. *J Clin Microbiol* **33**: 2826–2832.

Buwitt-Beckmann U, Heine H, Wiesmuller KH, Jung G, Brock R, Akira S & Ulmer AJ (2006) TLR1- and TLR6-independent recognition of bacterial lipopeptides. *J Biol Chem* **281**: 9049–9057.

Carr S, Penfold CN, Bamford V, James R & Hemmings AM (2000) The structure of TolB, an essential component of the tol-dependent translocation system, and its protein–protein interaction with the translocation domain of colicin E9. *Structure* **8**: 57–66.

Cascales E & Lloubes R (2004) Deletion analyses of the peptidoglycan-associated lipoprotein Pal reveals three independent binding sequences including a TolA box. *Mol Microbiol* **51**: 873–885.

Cascales E, Gavioli M, Sturgis JN & Lloubes R (2000) Proton motive force drives the interaction of the inner membrane TolA and outer membrane pal proteins in *Escherichia coli*. *Mol Microbiol* **38**: 904–915.

Cascales E, Lloubes R & Sturgis JN (2001) The TolQ-TolR proteins energize TolA and share homologies with the flagellar motor proteins MotA-MotB. *Mol Microbiol* **42**: 795–807.

Cascales E, Bernadac A, Gavioli M, Lazzaroni JC & Lloubes R (2002) Pal lipoprotein of *Escherichia coli* plays a major role in outer membrane integrity. *J Bacteriol* **184**: 754–759.

Chen R, Lim JH, Jono H *et al.* (2004) Nontypeable *Haemophilus influenzae* lipoprotein P6 induces MUC5AC mucin transcription via TLR2-TAK1-dependent p38 MAPK-AP1 and IKKbeta-IkappaBalpha-NF-kappaB signaling pathways. *Biochem Bioph Res Co* **324**: 1087–1094.

Clavel T, Lazzaroni JC, Vianney A & Portalier R (1996) Expression of the tolQRA genes of *Escherichia coli* K-12 is controlled by the RcsC sensor protein involved in capsule synthesis. *Mol Microbiol* **19**: 19–25.

Clavel T, Germon P, Vianney A, Portalier R & Lazzaroni JC (1998) TolB protein of *Escherichia coli* K-12 interacts with the outer membrane peptidoglycan-associated proteins Pal, Lpp and OmpA. *Mol Microbiol* **29**: 359–367.

Cordwell SJ, Len AC, Touma RG *et al.* (2008) Identification of membrane-associated proteins from *Campylobacter jejuni* strains using complementary proteomics technologies. *Proteomics* **8**: 122–139.

Delgado M, Yero D, Niebla O *et al.* (2007) Lipoprotein NMB0928 from *Neisseria meningitidis* serogroup B as a novel vaccine candidate. *Vaccine* **25**: 8420–8431.

Deprez C, Lloubes R, Gavioli M, Marion D, Guerlesquin F & Blanchard L (2005) Solution structure of the *E.coli* TolA C-terminal domain reveals conformational changes upon binding to the phage g3p N-terminal domain. *J Mol Biol* **346**: 1047–1057.

Derouiche R, Benedetti H, Lazzaroni JC, Lazdunski C & Lloubes R (1995) Protein complex within *Escherichia coli* inner membrane. TolA N-terminal domain interacts with TolQ and TolR proteins. *J Biol Chem* **270**: 11078–11084.

Derouiche R, Lloubes R, Sasso S, Bouteille H, Oughideni R, Lazdunski C & Loret E (1999) Circular dichroism and molecular modeling of the *E. coli* TolA periplasmic domains. *Biospectroscopy* **5**: 189–198.

Dubuisson JF, Vianney A & Lazzaroni JC (2002) Mutational analysis of the TolA C-terminal domain of *Escherichia coli* and genetic evidence for an interaction between TolA and TolB. *J Bacteriol* **184**: 4620–4625.

Dubuisson JF, Vianney A, Hugouvieux-Cotte-Pattat N & Lazzaroni JC (2005) Tol–Pal proteins are critical cell envelope components of *Erwinia chrysanthemi* affecting cell morphology and virulence. *Microbiology* **151**: 3337–3347.

Eick-Helmerich K & Braun V (1989) Import of biopolymers into *Escherichia coli*: nucleotide sequences of the exbB and exbD genes are homologous to those of the tolQ and tolR genes, respectively. *J Bacteriol* **171**: 5117–5126.

Ferrari G, Garaguso I, Adu-Bobie J *et al.* (2006) Outer membrane vesicles from group B *Neisseria meningitidis* delta gna33 mutant: proteomic and immunological comparison with detergent-derived outer membrane vesicles. *Proteomics* **6**: 1856–1866.

Fortney KR, Young RS, Bauer ME, Katz BP, Hood AF, Munson RS Jr & Spinola SM (2000) Expression of peptidoglycan-associated lipoprotein is required for virulence in the human model of *Haemophilus ducreyi* infection. *Infect Immun* **68**: 6441–6448.

Gaspar JA, Thomas JA, Marolda CL & Valvano MA (2000) Surface expression of O-specific lipopolysaccharide in *Escherichia coli* requires the function of the TolA protein. *Mol Microbiol* **38**: 262–275.

Gerding MA, Ogata Y, Pecora ND, Niki H & de Boer PA (2007) The trans-envelope Tol–Pal complex is part of the cell division machinery and required for proper outer-membrane invagination during cell constriction in *E. coli*. *Mol Microbiol* **63**: 1008–1025.

Germon P, Clavel T, Vianney A, Portalier R & Lazzaroni JC (1998) Mutational analysis of the *Escherichia coli* K-12 TolA N-terminal region and characterization of its TolQ-interacting domain by genetic suppression. *J Bacteriol* **180**: 6433–6439.

Gully D & Bouveret E (2006) A protein network for phospholipid synthesis uncovered by a variant of the tandem affinity purification method in *Escherichia coli*. *Proteomics* **6**: 282–293.

Heilpern AJ & Waldor MK (2000) CTXphi infection of *Vibrio cholerae* requires the tolQRA gene products. *J Bacteriol* **182**: 1739–1747.

Hellman J, Roberts JD Jr, Tehan MM, Allaire JE & Warren HS (2002) Bacterial peptidoglycan-associated lipoprotein is released into the bloodstream in gram-negative sepsis and causes inflammation and death in mice. *J Biol Chem* **277**: 14274–14280.

Henry T, Pommier S, Journet L, Bernadac A, Gorvel JP & Lloubes R (2004) Improved methods for producing outer membrane vesicles in Gram-negative bacteria. *Res Microbiol* **155**: 437–446.

Ihalin R, Karched M, Eneslatt K & Asikainen S (2006) Characterization of immunoaffinity purified peptidoglycan-associated lipoprotein of *Actinobacillus actinomycetemcomitans*. *J Chromatogr B* **831**: 116–125.

Journet L, Rigal A, Lazdunski C & Benedetti H (1999) Role of TolR N-terminal, central, and C-terminal domains in dimerization and interaction with TolA and tolQ. *J Bacteriol* **181**: 4476–4484.

Kadurugamuwa JL & Beveridge TJ (1995) Virulence factors are released from *Pseudomonas aeruginosa* in association with membrane vesicles during normal growth and exposure to gentamicin: a novel mechanism of enzyme secretion. *J Bacteriol* **177**: 3998–4008.

Kadurugamuwa JL & Beveridge TJ (1996) Bacteriolytic effect of membrane vesicles from *Pseudomonas aeruginosa* on other bacteria including pathogens: conceptually new antibiotics. *J Bacteriol* **178**: 2767–2774.

Kampfenkel K & Braun V (1993) Membrane topologies of the TolQ and TolR proteins of *Escherichia coli*: inactivation of TolQ by a missense mutation in the proposed first transmembrane segment. *J Bacteriol* **175**: 4485–4491.

Karched M, Ihalin R, Eneslatt K *et al.* (2008) Vesicle-independent extracellular release of a proinflammatory outer membrane lipoprotein in free-soluble form. *BMC Microbiol* **8**: 18.

Kim MJ, Sohn JW, Park DW, Park SC & Chun BC (2003) Characterization of a lipoprotein common to Legionella species as a urinary broad-spectrum antigen for diagnosis of Legionnaires' disease. *J Clin Microbiol* **41**: 2974–2979.

Kodama S, Hirano T, Suenaga S, Abe N & Suzuki M (2006) Eustachian tube possesses immunological characteristics as a mucosal effector site and responds to P6 outer membrane protein of nontypeable *Haemophilus influenzae*. *Vaccine* **24**: 1016–1027.

Konkel ME, Mead DJ & Cieplak W Jr (1996) Cloning, sequencing, and expression of a gene from *Campylobacter jejuni* encoding a protein (Omp18) with similarity to peptidoglycan-associated lipoproteins. *Infect Immun* **64**: 1850–1853.

Kuehn MJ & Kesty NC (2005) Bacterial outer membrane vesicles and the host–pathogen interaction. *Genes Dev* **19**: 2645–2655.

Lazzaroni JC & Portalier R (1992) The excC gene of *Escherichia coli* K-12 required for cell envelope integrity encodes the peptidoglycan-associated lipoprotein (PAL). *Mol Microbiol* **6**: 735–742.

Lazzaroni JC, Vianney A, Popot JL *et al.* (1995) Transmembrane alpha-helix interactions are required for the functional assembly of the *Escherichia coli* Tol complex. *J Mol Biol* **246**: 1–7.

Lazzaroni JC, Germon P, Ray MC & Vianney A (1999) The Tol proteins of *Escherichia coli* and their involvement in the uptake of biomolecules and outer membrane stability. *FEMS Microbiol Lett* **177**: 191–197.

Lee EY, Bang JY, Park GW *et al.* (2007) Global proteomic profiling of native outer membrane vesicles derived from *Escherichia coli*. *Proteomics* **7**: 3143–3153.

Levengood SK, Beyer WF Jr & Webster RE (1991) TolA: a membrane protein involved in colicin uptake contains an extended helical region. *P Natl Acad Sci USA* **88**: 5939–5943.

Liang MD, Bagchi A, Warren HS *et al.* (2005) Bacterial peptidoglycan-associated lipoprotein: a naturally occurring toll-like receptor 2 agonist that is shed into serum and has synergy with lipopolysaccharide. *J Infect Dis* **191**: 939–948.

Llamas MA, Ramos JL & Rodriguez-Herva JJ (2000) Mutations in each of the tol genes of *Pseudomonas putida* reveal that they are critical for maintenance of outer membrane stability. *J Bacteriol* **182**: 4764–4772.

Llamas MA, Ramos JL & Rodriguez-Herva JJ (2003a) Transcriptional organization of the *Pseudomonas putida* tol-oprL genes. *J Bacteriol* **185**: 184–195.

Llamas MA, Rodriguez-Herva JJ, Hancock RE, Bitter W, Tommassen J & Ramos JL (2003b) Role of *Pseudomonas putida* tol-oprL gene products in uptake of solutes through the cytoplasmic membrane. *J Bacteriol* **185**: 4707–4716.

Lloubes R, Cascales E, Walburger A, Bouveret E, Lazdunski C, Bernadac A & Journet L (2001) The Tol-Pal proteins of the *Escherichia coli* cell envelope: an energized system required for outer membrane integrity? *Res Microbiol* **152**: 523–529.

Lubkowski J, Hennecke F, Pluckthun A & Wlodawer A (1999) Filamentous phage infection: crystal structure of g3p in complex with its coreceptor, the C-terminal domain of TolA. *Structure* **7**: 711–722.

McMahon M, Murphy TF, Kyd J & Thanavala Y (2005) Role of an immunodominant T cell epitope of the P6 protein of nontypeable *Haemophilus influenzae* in murine protective immunity. *Vaccine* **23**: 3590–3596.

Muller MM & Webster RE (1997) Characterization of the tol–pal and cyd region of *Escherichia coli* K-12: transcript analysis and identification of two new proteins encoded by the cyd operon. *J Bacteriol* **179**: 2077–2080.

Murphy TF, Kirkham C & Lesse AJ (2006) Construction of a mutant and characterization of the role of the vaccine antigen P6 in outer membrane integrity of nontypeable *Haemophilus influenzae*. *Infect Immun* **74**: 5169–5176.

Neufert C, Pai RK, Noss EH, Berger M, Boom WH & Harding CV (2001) *Mycobacterium tuberculosis* 19-kDa lipoprotein promotes neutrophil activation. *J Immunol* **167**: 1542–1549.

Norskov-Lauritsen N & Kilian M (2006) Reclassification of *Actinobacillus actinomycetemcomitans, Haemophilus aphrophilus, Haemophilus paraphrophilus* and *Haemophilus*

segnis as *Aggregatibacter actinomycetemcomitans* gen. nov., comb. nov., *Aggregatibacter aphrophilus* comb. nov. and *Aggregatibacter segnis* comb. nov., and amended description of *Aggregatibacter aphrophilus* to include V factor-dependent and V factor-independent isolates. *Int J Syst Evol Micr* **56**: 2135–2146.

O'Hallahan J, Lennon D & Oster P (2004) The strategy to control New Zealand's epidemic of group B meningococcal disease. *Pediatr Infect Dis J* **12** (suppl): S293–S298.

Oscarsson J, Karched M, Thay B, Chen C & Asikainen S (2008) Proinflammatory effect in whole blood by free soluble bacterial components released from planktonic and biofilm cells. *BMC Microbiology* **8**.

Parsons LM, Lin F & Orban J (2006) Peptidoglycan recognition by Pal, an outer membrane lipoprotein. *Biochemistry* **45**: 2122–2128.

Parsons LM, Grishaev A & Bax A (2008) The periplasmic domain of TolR from *Haemophilus influenzae* forms a dimer with a large hydrophobic groove: NMR solution structure and comparison to SAXS data. *Biochemistry* **47**: 3131–3142.

Paul-Satyaseela M, Karched M, Bian Z *et al.* (2006) Immunoproteomics of *Actinobacillus actinomycetemcomitans* outer-membrane proteins reveal a highly immunoreactive peptidoglycan-associated lipoprotein. *J Med Microbiol* **55**: 931–942.

Pawelec DP, Korsak D, Wyszynska AK, Rozynek E, Popowski J & Jagusztyn-Krynicka EK (2000) Genetic diversity of the *Campylobacter* genes coding immunodominant proteins. *FEMS Microbiol Lett* **185**: 43–49.

Porcella SF & Schwan TG (2001) *Borrelia burgdorferi* and *Treponema pallidum*: a comparison of functional genomics, environmental adaptations, and pathogenic mechanisms. *J Clin Invest* **107**: 651–656.

Rau H, Revets H, Cornelis P, Titzmann A, Ruggli N, McCullough KC & Summerfield A (2006) Efficacy and functionality of lipoprotein OprI from *Pseudomonas aeruginosa* as adjuvant for a subunit vaccine against classical swine fever. *Vaccine* **24**: 4757–4768.

Ray MC, Germon P, Vianney A, Portalier R & Lazzaroni JC (2000) Identification by genetic suppression of *Escherichia coli* TolB residues important for TolB–Pal interaction. *J Bacteriol* **182**: 821–824.

Riechmann L & Holliger P (1997) The C-terminal domain of TolA is the coreceptor for filamentous phage infection of *E. coli*. *Cell* **90**: 351–360.

Sha J, Fadl AA, Klimpel GR, Niesel DW, Popov VL & Chopra AK (2004) The two murein lipoproteins of *Salmonella enterica* serovar Typhimurium contribute to the virulence of the organism. *Infect Immun* **72**: 3987–4003.

Sha J, Agar SL, Baze WB *et al.* (2008) Braun lipoprotein (Lpp) contributes to virulence of yersiniae: potential role of Lpp in inducing bubonic and pneumonic plague. *Infect Immun* **76**: 1390–1409.

Sturgis JN (2001) Organisation and evolution of the tol–pal gene cluster. *J Mol Microb Biotech* **3**: 113–122.

Takeuchi O, Kawai T, Muhlradt PF *et al.* (2001) Discrimination of bacterial lipoproteins by Toll-like receptor 6. *Int Immunol* **13**: 933–940.

Takeuchi O, Sato S, Horiuchi T *et al.* (2002) Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. *J Immunol* **169**: 10–14.

Valentine CH, Hellman J, Beasley-Topliffe LK, Bagchi A & Warren HS (2006) Passive immunization to outer membrane proteins MLP and PAL does not protect mice from sepsis. *Mol Med* **12**: 252–258.

Vianney A, Muller MM, Clavel T, Lazzaroni JC, Portalier R & Webster RE (1996) Characterization of the tol–pal region of *Escherichia coli* K-12: translational control of tolR expression by TolQ and identification of a new open reading frame downstream of pal encoding a periplasmic protein. *J Bacteriol* **178**: 4031–4038.

Vines ED, Marolda CL, Balachandran A & Valvano MA (2005) Defective O-antigen polymerization in tolA and pal mutants of *Escherichia coli* in response to extracytoplasmic stress. *J Bacteriol* **187**: 3359–3368.

Vipond C, Suker J, Jones C, Tang C, Feavers IM & Wheeler JX (2006) Proteomic analysis of a meningococcal outer membrane vesicle vaccine prepared from the group B strain NZ98/254. *Proteomics* **11**: 3400–3413.

Walburger A, Lazdunski C & Corda Y (2002) The Tol/Pal system function requires an interaction between the C-terminal domain of TolA and the N-terminal domain of TolB. *Mol Microbiol* **44**: 695–708.

Webster RE (1991) The tol gene products and the import of macromolecules into *Escherichia coli*. *Mol Microbiol* **5**: 1005–1011.

Wyszynska A, Pawelec DP & Jagusztyn-Krynicka EK (2002) Immunological characterization of the *Campylobacter jejuni* 72Dz/92 cjaD gene product and its fusion with B subunit of E. coli LT toxin. *Acta Microbiol Pol* **51**: 313–326.

Yamauchi K, Hotomi M, Billal DS, Suzumoto M & Yamanaka N (2006) Maternal intranasal immunization with outer membrane protein P6 maintains specific antibody level of derived offspring. *Vaccine* **24**: 5294–5299.

Yoon WS, Park SH, Park YK, Park SC, Sin JI & Kim MJ (2002) Comparison of responses elicited by immunization with a *Legionella* species common lipoprotein delivered as naked DNA or recombinant protein. *DNA Cell Biol* **21**: 99–107.

Zhai YF, Heijne W & Saier MH (2003) Molecular modeling of the bacterial outer membrane receptor energizer, ExbBD/TonB, based on homology with the flagellar motor, MotAB. *Biochim Biophys Acta* **1614**: 201–210.

Zhang XY, Goemaere EL, Thome R, Gavioli M, Cascales E & Lloubes R (2008) Mapping the interactions between *Escherichia coli* Tol subunits. Rotation of the TolR transmembrane helix. *J Biol Chem* **284**: 4275–4282.

Zhuang Z, Song F, Martin BM & Dunaway-Mariano D (2002) The YbgC protein encoded by the ybgC gene of the tol–pal gene cluster of *Haemophilus influenzae* catalyzes acyl-coenzyme A thioester hydrolysis. *FEBS Lett* **516**: 161–163.